

**Environmental Effects on Brain Estrogen Receptor
Expression and Aggression**

A Senior Honors Thesis

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By

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Abstract

It is broadly accepted that the environment influences the effects of genes on behavior, but the mechanisms mediating these environmental effects on phenotype are poorly defined. The present study examined whether photoperiod (day length) and reproductive experience, two important environmental variables, affect gene expression to influence aggressive behavior. Individuals respond to photoperiod because it predicts important variability in the environment; male rodents use photoperiod to time adaptive behaviors such as mating and aggression. For example, mating is more likely in rodents housed in long, summer-like days when testosterone concentrations are high, whereas aggression in some rodent species is paradoxically elevated when housed in short, winter-like days when testosterone concentrations are low. Previous work in *Peromyscus polionotus* indicated that brain estrogen receptor alpha (ER α) expression is increased in short days (8L:16D), whereas brain estrogen receptor beta (ER β) expression is increased in long days (16L:8D). Hormone manipulation studies suggested that the photoperiodic effect on aggression occurs independently of changes in ER expression. This hypothesis was tested directly by examining the effects of photoperiod on aggression and ER expression in monogamous *P. californicus*, which do not reduce testes size in short days. I also examined how aggression changes in relation to parental behavior. Nulliparous male *P. californicus* were significantly more aggressive when housed in short versus long days, and parental males were also significantly more aggressive than nulliparous mice kept in long days. Neither photoperiod nor reproductive experience affected the expression of either ER α or ER β in brain nuclei that are components of the brain “social behavior network.” These results suggest that the effects of photoperiod and reproduction

on aggression are independent of changes in ER expression. Additionally, these data emphasize the importance of studying the biological mechanisms mediating aggression under different environmental conditions in order to better understand the neurobiological bases of this complex social behavior.

Introduction

Aggression and violence are important problems in our society. Although the word ‘aggression’ has many definitions, a useful behavioral definition is an “overt behavior with the intention of inflicting physical damage upon another individual” (Nelson, 2006). The field of human and animal research on aggression and violence is rapidly growing; in general, the goal of researchers has been to prevent unjustifiable aggressive behavior from occurring (Lederhendler, 2003). Any phenotype, including behavior, is the result of an interaction between genes and the environment. Although there has been much research on the effects of genes on behavior, less is known about how environmental factors can regulate the genes that influence behavior.

Consequently, it is important to study the biological mechanisms involved in aggression under different environmental conditions in order to more fully understand the neurobiological basis of this problematic behavior. I tested this by using a simple, quantifiable environmental variable (i.e., the number of hours of light per day), as well as a complex social environmental variable (i.e., reproductive and parental experience) to evaluate these environmental effects on gene expression in neural circuits involved in aggression.

Environmental conditions vary predictably across the year. This has led to the evolution of several physiological adaptations that allow organisms to synchronize energetically expensive processes to changes in the environment. Most small mammals breed during the spring and early summer when conditions are more benevolent and food is abundant; during other times in the year, energetic investments are biased towards processes that help the organism survive until the next breeding season (Prendergast,

Nelson, & Zucker, 2002). In order to be ready for changes in the environment, many organisms use photoperiodic (day length) information to predict the changing seasons. Photoperiod is an environmental cue that can provide specific and accurate information about the time of the year. In the laboratory, manipulating day length can induce the processes associated with the changing seasons. In most vertebrates, photoperiod information is transduced from an environmental factor to a physiological signal via the duration of nighttime secretion of melatonin (Goldman, 2001).

Researchers have not yet examined whether photoperiod and reproductive experiences, two potentially significant environmental variables, affect the same neural systems to influence aggressive behavior. One way that these experiences could influence aggression is via altering the effect of estrogens on behavior. Estrogenic hormones act differently on aggressive behaviors in different species. Previous research has shown that the way estrogens influence aggression in mice is context-dependent (Trainor, Lin, Finy, Rowland, & Nelson, 2007).

The effects of estrogens are dependent on the type of estrogen receptor (ER) to which the hormone binds. There are two well described ER subtypes: ER α and ER β . Male ER α knockout mice are less aggressive than male wild-type mice (Ogawa, Nomura, Choleris, & Pfaff, 2006). Male ER β knockout mice are more aggressive than male wild-type mice (Ogawa et al, 2006). Thus, it would seem that ER α promotes aggression in most male lab mice, whereas ER β inhibits aggression.

However, environmental factors can also affect the expression of estrogen receptors. In beach mice (*Peromyscus polionotus*) housed in short days (light exposure for eight hours, darkness exposure for sixteen hours a day) that resemble winter-like

photoperiods, injections of estradiol (one of the primary estrogens) increase aggression within fifteen minutes compared to saline injections (Trainor et al, 2007). In contrast, there is no difference in aggression among these mice housed in summer-like long days (light exposure for sixteen hours, darkness exposure for eight hours), injected with either estradiol or saline, then tested fifteen minutes later (Trainor et al, 2007). Thus, photoperiod influences how this hormone affects aggression in *P. polionotus*, acting through non-genomic mechanisms to increase aggression in short days but not in long days. Steroid hormones, such as estrogens, activate genes to affect behavior via genomic and non-genomic mechanisms. Genomic actions require binding of steroid hormones to intracellular receptors that are translocated to the cell nucleus, where the steroid-receptor complex binds to the DNA and acts as a gene expression activator or suppressor. Thus, genomic steroid actions typically take days or weeks to occur; in contrast, non-genomic effects of steroids, which typically involve binding to membrane receptors that activate signal transduction pathways, can occur within seconds or minutes (Stormshak & Bishop, 2007; Trainor et al, 2007). Therefore, because the rapid effects of estradiol injections occurred within fifteen minutes, there was not enough time for the traditional genomic effects to have occurred (Vasudevan & Pfaff, 2006).

Male *P. polionotus* also have small testes and reduced testosterone concentrations when they are housed in short-day environments compared to long-day environments (Trainor, Martin, Kuhlman, Greiwe, & Nelson, 2006). Maintaining a fully functional reproductive system is energetically expensive, so shutting off the reproductive system during short days helps individuals free up more energy for survival mechanisms (Nelson & Demas, 2004).

Previously our lab has reported that photoperiod and estrogen receptors interact to regulate aggressive behaviors. In *P. polionotus*, ER α expression is increased in short days (when animals are more aggressive), whereas ER β expression is increased in long days (when animals are less aggressive) (Trainor et al, 2007). Additionally, photoperiodic regulation of ER expression is responsible for changes in aggression. Both ER subtype selective agonists increase aggression in short-day mice and decrease aggression in long-day mice (Trainor et al, 2007). Apparently, photoperiod affects aggressive behavior by altering processes that occur after estrogens bind to the receptor and not through the differential expression of ER α or ER β . In other words, changes in ER subtype expression cannot explain the photoperiodic changes in aggression.

The pattern in several rodent species, such as *P. polionotus*, is to exhibit aggression in short-day environments and reduce the size of their testes. However, California mice (*Peromyscus californicus*) differ from closely related *P. polionotus* by not reducing their testes size in short-day environments (Nelson, Gubernick, & Blom, 1995) and by forming monogamous pairs in the wild (Ribble, 1991). Therefore, *P. californicus* are more similar to humans than many other rodents, making them an appropriate study species. Nevertheless, estrogens inhibit aggressive behavior in long-day California mice as this hormone does in *P. polionotus* (Trainor, Bird, & Marler, 2004). This suggests that even though *P. californicus* do not respond to short days with gonadal regression, the way estrogens influence aggression may still be regulated by photoperiod.

Changes in aggression are also observed with parental behavior in *P. californicus*, as male California mice become more aggressive when they become parents. Male

California mice take an active role in caring for their pups, and aromatase activity increases in their brains when they become fathers (Trainor, Bird, Alday, Schlinger, & Marler, 2003). Aromatase activity is important because androgenic hormones are converted into estrogens in the brain by aromatase enzymes.

Although it is known that both photoperiod and parental experience regulate aggression in *Peromyscus* mice, it is unknown whether these processes occur by the same neural mechanisms. That is, can the photoperiodic effects on aggression due to estrogen receptors be dissociated from reproductive responses in California mice? In this experiment, I will compare the differential contributions of two types of environmental conditions on aggressive behaviors and ER regulation. Nulliparous (an individual who has never given birth to offspring) males housed in both long and short photoperiods, and mice that have fathered at least two litters will be compared. I expect that the fathers in long-day environments will be more aggressive than nulliparous mice because aggression may protect pups from other males; furthermore, I predict that short-day nulliparous mice will be more aggressive than the long-day nulliparous mice. Paternal behavior and photoperiod should affect the expression of both ER α and ER β for *P. californicus*, and these environmental variables should mediate the effects of estrogens on behavior. There will likely not be differences in the number of ER subtypes in brain structures that compose the social behavior network of the fathers as compared to the nulliparous mice, and that there will be no differences in ER expression due to photoperiod in the nulliparous mice.

Methods

Experimental Design

This experiment used a between-subjects design. The independent variable was which of three experimental conditions the mice were randomly assigned to: nulliparous male mice housed in short-day environments, nulliparous male mice housed in long days, and male parental mice housed in long days. There were no paternal mice housed in short-day environments because this is an artificial condition and does not mimic anything in the field. Even though, theoretically, California mice are able to breed during short days, they only breed when green vegetation is present (Nelson et al, 1995). The dependent measures are aggressive behaviors and ER immunoreactivity in the neural components of the aggression brain circuitry including the lateral septum, medial preoptic area, bed nucleus of the stria terminalis (BNST), ventral BNST, medial amygdala, paraventricular nucleus (PVN) of the hypothalamus, and the ventral medial hypothalamus.

Animals

The animals used in this study were eighteen *P. californicus* males of approximately one year of age. The mice were obtained from a breeding colony maintained by Dr. Catherine Marler at the University of Wisconsin. Six of the males were individually housed and randomly assigned to be kept in long days (16L:8D), six males were individually housed and randomly assigned to be kept in short days (8L:16D), and six of the males were randomly assigned to be pair-housed with a female in a long-day environment.

Materials

All mice were housed in polypropylene cages (dimensions: 27.8 x 7.5 x 13 cm) during the experiment in temperatures around 24° Celsius (C). Animals were given unlimited access to phytoestrogen-free food (Harlan Teklad 2016) and filtered tap water. Intruder mice and video equipment were used for the aggression tests. Supplies and reagents were used for immunocytochemistry procedures. In addition, a Nikon E800 microscope with photographic capabilities was used to photograph immunoreactive cells in the desired brain regions.

Procedure

For the first part of this experiment, resident-intruder aggression tests were done on California mice to compare the aggressiveness of nulliparous male mice kept in short-day environments, nulliparous male mice kept in long days, and male parents kept in long days. The mice were kept in each photoperiod treatment group for eight weeks, and paternal mice were tested after they had weaned at least one litter and an additional litter had reached 2-3 weeks of age. Each mouse had an intruder California mouse put into its cage for seven minutes, and their aggressive interaction was filmed. Later, an observer uninformed about treatment conditions scored the amount of biting and the latency to attack.

After the behavioral tests, the mice were anesthetized with isoflurane and decapitated. The brains were immersed in a 5% acrolein/95% phosphate buffered saline (PBS) solution for 24 hours while kept at 4° C and were then transferred to a 30% sucrose/70% PBS solution for 24 hours while kept at 4° C. Then, the brains were frozen on dry ice and stored at 80° C.

The brains were later sectioned at 40 microns, and every third free-floating section kept in PBS was processed for either ER α or ER β immunocytochemistry. The sections were incubated in 1% sodium borohydride for ten minutes, rinsed in 20% normal goat serum and 0.3% hydrogen peroxide in PBS for twenty minutes, and then were incubated in either primary ER α (C1355, Upstate Biotechnology, 1:20K) or primary ER β (D7N, Invitrogen, 1:400) in 1% normal goat serum in 0.5% Triton-X PBS for 48 hours at 4° C. Afterward, the sections were rinsed three times in PBS for five minutes and incubated in biotinylated goat-anti-rabbit antibody (Vector Laboratories, 1:500) in 0.5% Triton-X PBS for two hours. The sections were then rinsed three times in PBS for five minutes and incubated for thirty minutes in avidin-biotin complex (ABC Elite kit, Vector Laboratories). After three more rinses in PBS for five minutes, the sections were developed in diaminobenzidine for two minutes. The sections were later rinsed two times in PBS for five minutes, mounted on gel-coated slides, dehydrated, and coverslipped with Permount.

The amount of ER expression in these mice was examined using immunocytochemistry staining to count immunoreactive cells in many brain structures with estrogen receptors that compose the social behavior network. These brain areas include the lateral septum, medial preoptic area, BNST, ventral BNST, medial amygdala, PVN of the hypothalamus, and the ventral medial hypothalamus. A Nikon E800 microscope with photographic capabilities was used to take photomicrographs by a program called PictureFrame for counting immunoreactive cells. The Ohio State University Institutional Laboratory Animal Care and Use Committee approved all experimental procedures.

Behavioral data and ER expression were analyzed with one-way between groups analyses of variance (ANOVA), and multiple comparisons were conducted with Fisher's protected LSD (Least Significant Difference). All comparisons were considered statistically significant when $p < 0.05$.

Results

Nulliparous males were significantly more aggressive when housed in short-day environments (15.29 ± 3.22 bites) compared to long days (6.44 ± 2.44 bites), and male parents were also significantly more aggressive (20.67 ± 3.97 bites) than nulliparous mice kept in long days (Figure 1A). In other words, long-day paternal and short-day nulliparous mice were much more aggressive than long-day nulliparous mice, $F(1,2) = 5.51, p < 0.05$. Nulliparous males also had significantly shorter attack latencies when housed in short days (35.25 ± 15.35 seconds) compared to long days (279.20 ± 88.00 seconds), and male parents were also significantly more aggressive (69.77 ± 34.88 seconds) than nulliparous mice kept in long days (Figure 1B). In other words, long-day paternal and short-day nulliparous mice had much shorter attack latencies than nulliparous long-day mice, $F(1,2) = 4.41, p < 0.05$.

Immunoreactive cells of ER α and ER β in the lateral septum, medial preoptic area, BNST, ventral BNST, medial amygdala, PVN of the hypothalamus, and the ventral medial hypothalamus were also counted. For the most part, the data demonstrate no differences in the number of immunoreactive cells among the brain structures examined for mice in all three treatment groups (Supplemental Material: Tables 1-11). However, ER α differed significantly in the medial preoptic area between paternal (208.17 ± 18.01

cells) and short-day nulliparous mice (278.20 ± 14.41 cells). Thus, paternal mice have significantly fewer immunoreactive cells stained for $ER\alpha$ in the medial preoptic area than nulliparous mice housed in short-day environments, $F(2,16) = 4.04$, $p < 0.05$.

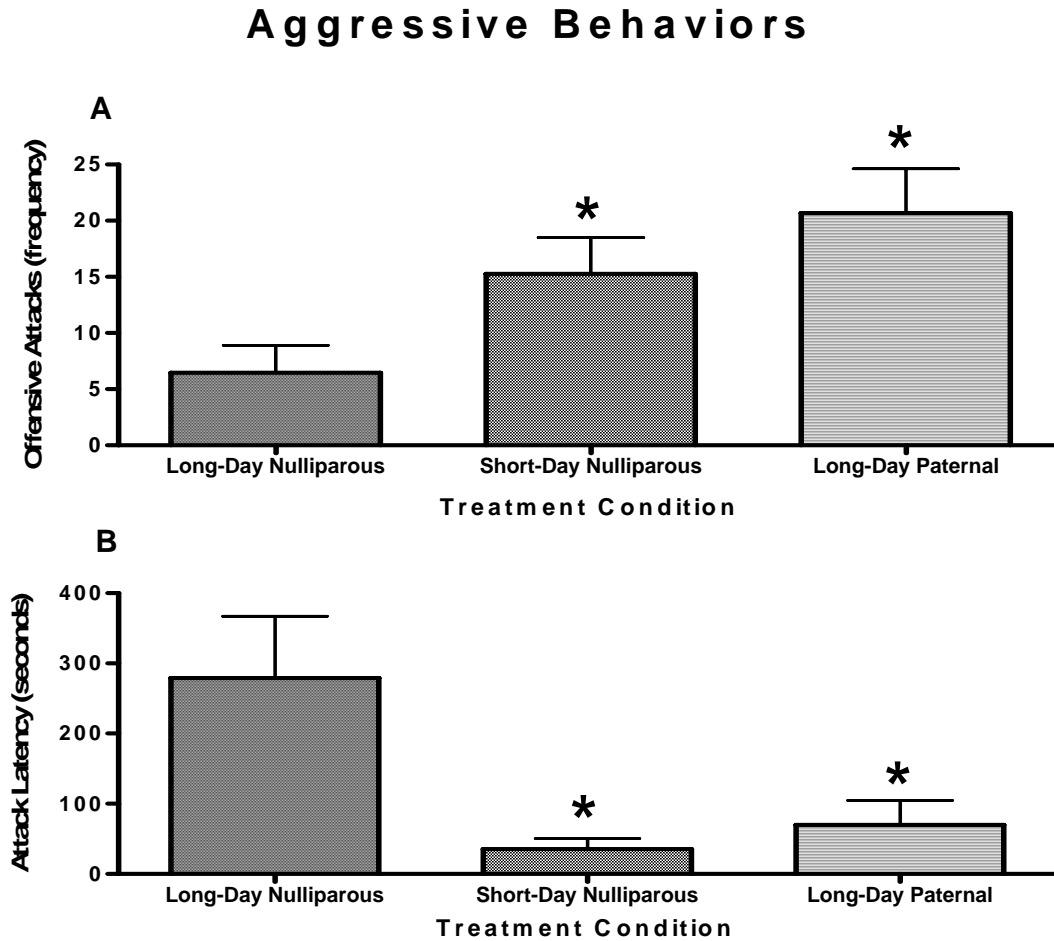


Figure 1: Aggressive behaviors among long-day nulliparous, short-day nulliparous, and long-day paternal mice in resident-intruder aggression tests: A) the frequency of offensive attacks (number of bites) and B) latency to attack in seconds. Short-day nulliparous and long-day paternal mice had greater offensive attacks and shorter attack latencies than long-day nulliparous mice but did not differ significantly from each other. Six mice per treatment group, $*p < 0.05$.

Discussion

These results support previous studies that demonstrated that photoperiod affects aggressive behaviors in male California mice. However, this is the first study in male California mice that has ever directly tested how parental experience affects aggression. Long-day paternal and short-day nulliparous mice displayed a higher frequency of offensive attacks and shorter attack latencies than long-day nulliparous mice. Although these two environmental factors affected aggressive behavior, these effects cannot be attributed to the differential expression of estrogen receptors. In other words, these behavior differences are not due to the up-regulation or down-regulation of estrogen receptors.

Taken by itself, it would appear that estrogens are not mediating these differences in aggressive behavior in this experiment. However, these results support hormone manipulation studies in *P. polionotus* (Trainor et al, 2007) that demonstrated either that estrogen receptors seem to be responsible for different functions under different contexts or that the effects of estrogens are mediated by non-estrogen receptor mechanisms. Testosterone concentrations were equivalent among treatment groups, which suggests that estrogenic hormones (which are produced by enzymatic modification of testosterone and other androgens) regulate estrogen receptors.

Nevertheless, the importance of gene-environment interactions cannot be overly emphasized. Future studies should look more at the processes that occur after estrogens bind to the receptor and the specific neurochemical pathways mediating the effects of aggressive behavior under different environmental conditions in California mice. Because males are more aggressive than females in most species that have been studied,

they are appropriate models to use when studying aggression (Nelson & Chiavegatto, 2001). However, it would still be valuable to replicate this experiment with female California mice to examine maternal nest defensive behaviors.

There are several limitations to this study. Because there were no paternal animals housed in short-day environments, the effects of photoperiod on parental experience cannot be completely separated. There were also only six mice per treatment group, which is a relatively small sample size. An additional confounding variable in this study is that the nulliparous mice were individually housed but the long-day parental mice were pair-housed with a female until ten minutes before the behavior tests. Consequently, these limitations should be addressed by future studies.

The only difference between treatment groups in any of the examined brain structures is of ER α in the medial preoptic area between long-day paternal and short-day nulliparous mice. In this brain structure, paternal mice have less immunoreactive cells stained for ER α than nulliparous mice housed in short-day environments. Previous studies have reported that male California mice have more aromatase activity in their brains when they have offspring (Trainor et al, 2003), and so a possible explanation for this difference may be due to increased aromatase activity contributing to negative feedback in the medial preoptic area of the paternal mice. The medial preoptic area is an important brain structure in the context of maternal aggression (Numan, 2007), and so it is worthy to note that this brain area had less ER α cells. It has been demonstrated that lesions to the medial preoptic area disrupt parental behavior in both male and female California mice (Lee & Brown, 2002), and so less ER α cells could also mean that this brain structure is supersensitive to circulating estrogens.

These results indicate that environmental factors affect aggressive behaviors. Photoperiod (a simple, quantifiable environmental variable) and paternal experience (a complex social environmental variable) affected neural circuits involved in regulating aggression. However, the literature on the molecular basis of aggressive behavior is very extensive, and there are many known neurotransmitters, hormones, cytokines, enzymes, growth factors, and signaling molecules that affect aggression (Nelson & Chiavegatto, 2001). Therefore, other neural mechanisms important in regulating this complex social behavior must also be studied to gain a better understanding of aggression.

The potential significance of these findings will be a valuable contribution to the growing literature on the complicated role of estrogen receptors in aggression while giving us more of an insight into the biological mechanisms of this complex behavior. There are also many practical applications from the results of this experiment. Psychological states, such as mood and depression, and behavioral processes, including criminal behavior, vary seasonally in humans, and this study helps to shed light on some of the gene-environment interactions important in influencing these behaviors. By understanding the effects of estrogens on aggressive behavior in mice, we will be better able to understand the mechanisms that affect components of aggression and hostility in humans.

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Supplemental Material: ER α and ER β Immunoreactive Cells in the Social Behavior Network

Table 1: ER α in Lateral Septum

	Mean	SE
Long-Day Nulliparous Mice	133.33	9.61
Short-Day Nulliparous Mice	142.00	31.14
Long-Day Paternal Mice	142.50	16.52

Table 2: ER α in Medial Preoptic Area

	Mean	SE
Long-Day Nulliparous Mice	255.67	19.11
Short-Day Nulliparous Mice	278.20*	14.41
Long-Day Paternal Mice	208.17*	18.01

Table 3: ER β in Medial Preoptic Area

	Mean	SE
Long-Day Nulliparous Mice	99.83	44.35
Short-Day Nulliparous Mice	161.20	30.08
Long-Day Paternal Mice	94.20	15.76

Table 4: ER α in BNST

	Mean	SE
Long-Day Nulliparous Mice	183.83	27.03
Short-Day Nulliparous Mice	177.80	35.95
Long-Day Paternal Mice	155.67	45.35

Table 5: ER β in BNST

	Mean	SE
Long-Day Nulliparous Mice	284.00	39.65
Short-Day Nulliparous Mice	267.00	42.53
Long-Day Paternal Mice	219.33	25.02

Table 6: ER α in ventral BNST

	Mean	SE
Long-Day Nulliparous Mice	43.17	6.87
Short-Day Nulliparous Mice	57.60	14.40
Long-Day Paternal Mice	45.00	8.08

Table 7: ER α in Medial Amygdala

	Mean	SE
Long-Day Nulliparous Mice	57.17	22.40
Short-Day Nulliparous Mice	118.60	47.86
Long-Day Paternal Mice	89.67	28.49

Table 8: ER β in Medial Amygdala

	Mean	SE
Long-Day Nulliparous Mice	211.67	57.96
Short-Day Nulliparous Mice	269.60	70.24
Long-Day Paternal Mice	236.33	31.65

Table 9: ER α in PVN

	Mean	SE
Long-Day Nulliparous Mice	63.50	19.41
Short-Day Nulliparous Mice	66.80	12.19
Long-Day Paternal Mice	61.00	15.07

Table 10: ER β in PVN

	Mean	SE
Long-Day Nulliparous Mice	49.83	9.07
Short-Day Nulliparous Mice	57.25	4.31
Long-Day Paternal Mice	32.83	7.16

Table 11: ER α in Ventral Medial Hypothalamus

	Mean	SE
Long-Day Nulliparous Mice	157.83	12.79
Short-Day Nulliparous Mice	131.67	13.69
Long-Day Paternal Mice	143.50	11.64

Supplemental Material: Tables 1-11 above show the mean and standard error (SE) of estrogen receptor (ER) immunoreactivity in the lateral septum (Table 1), medial preoptic area (Tables 2 & 3), bed nucleus of the stria terminalis (BNST) (Tables 4 & 5), ventral BNST (Table 6), medial amygdala (Tables 7 & 8), paraventricular nucleus (PVN) of the hypothalamus (Tables 9 & 10), and the ventral medial hypothalamus (Table 11).

Immunoreactive cells of ER β in the lateral septum, ventral BNST, and ventral medial hypothalamus were not counted because there are no ER β cells present in these brain

structures in California mice. Six mice per treatment group, all p 's > 0.05 , except for ER α in the medial preoptic area.

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